INTRAUTERINE GROWTH RESTRICTION SEX-SPECIFICALLY ALTERS SEX-STEROID SIGNALING IN RAT LUNG

by

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ABSTRACT

Intrauterine growth restriction (IUGR) refers to the failure of a fetus to reach its genetic growth potential *in utero*. Each year in the United States, 5-12% of premature babies are born IUGR, which increases their risk for postnatal morbidities. One such postnatal morbidity is brochopulmonary dysplasia (BPD), in which males are more severely affected than females. Histologically, BPD is characterized by impaired alveolar development. One pathway contributing to alveolar formation is estrogen signaling.

The predominate estrogen in the lung, estradiol, binds to estrogen receptors (ERs) in the cytosol, dimerizes, and translocates to the nucleus. In the nucleus, ERs bind to estrogen response elements on target genes and affect transcription, resulting in appropriate gene expression and lung development. Androgen signaling works in a similar way through the ligand testosterone and an androgen receptor (AR). Testosterone within the lung can be used to produce estradiol locally by the converting enzyme aromatase. Normal alveolar formation requires an appropriate estrogen to testosterone ratio.

We hypothesize that IUGR alters lung sex steroids, estradiol to testosterone ratios, and ER/AR expression in a sex-specific manner in newborn rat lung.

Uteroplacental insufficiency was induced in pregnant rats by uterine artery

ligation on day 19 of gestation, producing IUGR pups. We examined 4 groups for this study: IUGR female, IUGR male, control female, and control male. Each group consisted of *n=6* rat pups derived from different litters. ELISA was used to measure protein concentrations of serum and lung estradiol and testosterone, while Western blotting was used to determine ERα, ERβ, and AR protein abundance relative to GAPDH.

The serum and lung estrogen to testosterone ratios as well as ERα protein abundance is depressed in male but not female newborn IUGR rat pups when compared to sex-matched controls. ERβ and AR expression are increased in IUGR males when compared to sex-matched controls. We conclude that IUGR alters lung sex steroids, estradiol to testosterone ratios, and ER/AR expression in a sex-specific manner in newborn rat lung. We speculate that depressed estrogen signaling in male IUGR lung may contribute to worse outcomes.

TABLE OF CONTENTS

ABSTRACT iii

LIST OF FIGURES..... vi

ACKNOWLEDGEMENTS..... vii

INTRODUCTION..... 1

METHODS..... 4

RESULTS..... 6

DISCUSSION..... 13

REFERENCES..... 15

LIST OF FIGURES

Figure Page

1. Serum estradiol..... 8

2. Serum testosterone..... 8

3. Serum estradiol to testosterone ratio..... 9

4. Lung estradiol..... 9

5. Lung testosterone..... 10

6. Lung estradiol to testosterone ratio..... 10

7. ERα protein abundance..... 11

8. ERβ protein abundance..... 11

9. AR protein abundance..... 12

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INTRODUCTION

Intrauterine growth restriction (IUGR) refers to the failure of a fetus to reach its genetic growth potential *in utero*. Each year in the United States, 5-12% of premature babies are born IUGR, which increases their risk for postnatal morbidities (Hoyert, 2004). Risk factors for IUGR include maternal diabetes, chronic hypertension, preeclampsia, smoking, alcohol ingestion, and poor diet (Gaudineau, 2013). Common problems experienced by IUGR infants include lung function impairment, underdeveloped GI tracts and feeding problems, cognitive delays, and neurosensory deficits (Joss- Moore, 2013).

A postnatal morbidity that affects many IUGR infants is brochopulmonary dysplasia (BPD), with male infants being more severely affected than females (Regev, 2013; Reiss, 2003; Stein, 1997). Histologically, BPD is characterized by alveolar simplification, or impaired alveolar development (Bhandari, 2003; Bhatt, 2001; Coalson, 2006). Alveolar formation is the last stage of lung development, and involves the transition from the saccular stage to the alveolar stage. During the saccular stage of development, the lung parenchyma is composed of saccules lined by cuboidal epithelial cells, which contribute to the production of surfactant. These saccules form secondary septa and subdivide into alveoli. This transformation requires saccule wall thinning via apoptosis as well as capillary growth and expansion into the epithelial cells. Simultaneously, alveolar type 2 epithelial cells

synthesize and secrete surfactant (Burri, 1984, 2006). Sex-steroid signaling is an important component of alveolar formation.

Sex-steroid signaling in the lung includes estrogen signaling and androgen signaling. In general, estrogen signaling has a positive effect on alveolar formation, while androgen signaling can be inhibitory under some circumstances. The primary estrogen in the lung is estradiol. Estradiol activity is propagated via estrogen receptors, including estrogen receptors alpha and beta, which I will abbreviate as ERα and ERβ. ERs are nuclear receptor transcription factors that can positively or negatively modulate the transcription of target genes (Hall 2001). The primary androgen in the lung is testosterone (Heinlin, 2002). Test osterone activity is propagated via androgen receptors (AR). ARs mostly positively modulate the transcription of target genes (He, 1999; Heinlein, 2002; Quigley, 1998; Roy , 1999).

In both estrogen signaling and androgen signaling, the ligand (estradiol or testosterone), binds to its sex-steroid receptor in the cytosol (ERα, ERβ, or AR), dimerizes, and translocates to the nucleus. In the nucleus, the sex-steroid receptors bind to response elements on target genes and affect transcription, resulting in appropriate gene expression and lung development.

Estradiol within the lung can originate from circulating estradiol. Alternatively, estradiol within the lung can be locally produced. Estradiol is produced within the lung from testosterone via the enzyme aromatase (Homma, 2015). While estradiol is important in normal alveolar formation, the estrogen to testosterone ratio may be a stronger factor in alveolar formation (Seaborn, 2010).

IUGR male lungs exhibit developmental delays when compared to IUGR

female lungs (Joss- Moore, 2013). The delay in male lung maturation may be androgen-related since androgens hinder the surge of surfactant lipid production that accompanies alveolar formation in the lung (Bresson, 2010; Nielsen, 1985). Previous data from our lab demonstrated that IUGR alters the expression of the metabolizing enzyme aromatase in a sex-divergent manner (Stiers, 2013). We also know that phenotypically, IUGR females do better than IUGR males, especially after an additional postnatal stressor (Joss- Moore, 2013). This is true in humans and in rat models of IUGR.

The effect of IUGR on serum estradiol and testosterone, lung estradiol and testosterone, and ERα/ERβ/AR expression is currently unknown. We hypothesize that IUGR alters lung sex steroids, estradiol to testosterone ratios, and ER/AR expression in a sex-specific manner in newborn rat lung.

METHODS

Uteroplacental insufficiency was induced in pregnant Sprague-Dawley rats by bilateral uterine artery ligation on day 19 of gestation, producing IUGR pups. Both IUGR and control dams underwent identical anesthetic procedures. Newborn, or day 0 (d0), rat pups were delivered via caesarian section at term, 2.5 days after the artery ligation surgery. Rat pups were killed by decapitation and blood collected for serum separation. Lungs were immediately removed from the animals, flash-frozen in liquid nitrogen, and stored at -80 degrees C.

We examined 4 groups for this study: IUGR female, IUGR male, control female, and control male. Each group consisted of $n=6$ rat pups derived from different litters.

Harvested d0 rat lungs were crushed using mortar and pestle with liquid Nitrogen keeping samples cold. A RIPA buffer was used to lyse cells to allow for protein extraction. Pierce BCA protein Assay Reagents A and B were used to generate a standard curve and measure protein concentration within each sample.

We measured estradiol and testosterone levels in serum and lung tissue using an estradiol ELISA kit from Alpha Diagnostics. The experiments were run according to manufacturer instructions.

Western blot was used to measure the abundance of ERα, ERβ, and AR

protein in male and in female IUGR and control d0 rat lungs. The antibodies used were ERα rabbit polyclonal IgG (H-184):sc-7207 from Santa Cruz Blotechnology, ERβ rabbit polyclonal ab16813 from abcam, AR rabbit polyclonal ab74272 from abcam, GAP-DH (14C10) rabbit (#2118L) from Cell Signaling , and Anti-rabbit IgG HRP- linked (#7074S) from Cell Signaling.

We loaded 20 micrograms of sample on an 8% Bis-Tris polyacrylamide midi gel in a MOPS buffer for electrophoresis. Following electrophoresis for 1 hour at 140V, the gel was transferred at 100V for 1 hour while on ice. The blocking agent was 5% milk, the washing solution was TBS-T1%, and the overnight protocol was used on a Blot Cycler.

Data are expressed as the mean ± standard deviation *(SD)*. ANOVA was used to compare all 4 study groups simultaneously. Statistical significance was accepted at $p \leq 0.05$.

RESULTS

We first examined serum estradiol levels. Serum estradiol levels are significantly higher in male control than in female control newborn rat pups *(p= 0.0002)*. IUGR decreases serum estradiol levels in newborn male rat pups *(p= 0.0019)*, but not in female rat pups (Figure 1). Next we tested serum testosterone levels. Serum testosterone levels are higher in female control than in male control newborn rat pups *(p=0.0011).* IUGR does not change serum testosterone levels in newborn male or female rat pups (Figure 2). Combining the serum estradiol and testosterone data just presented, male control serum estradiol to testosterone ratio is significantly higher than female control *(p= <0.001).* The serum estradiol to testosterone ratio decreases in male *(p= 0.0019)* but not female IUGR newborn rat pups when compared to sex-matched controls (Figure 3).

We next examined lung estradiol levels. Lung estradiol levels are similar in male control and female control newborn rat pups. IUGR increases lung estradiol levels in newborn female rat pups *(p= 0.0404),* but not in male rat pups (Figure 4). The following data set is for lung testosterone levels. Lung testosterone levels are similar in male control and female control newborn rat pups. IUGR increases lung testosterone levels in newborn male rat pups *(p=0.0338),* but not in female rat pups (Figure 5). We again combine estrogen and testosterone data to present ratios for

control and IUGR rat pups. Lung estradiol to testosterone ratio increases in female *(p= 0.0239)* and decreases in male *(p= 0.05)* IUGR newborn rat pups when compared to sex-matched controls (Figure 6).

We examined lung ERα protein abundance relative to GAPDH. Lung ERα protein abundance is higher in male control than in female control newborn rat pups *(p= 0.0123).* IUGR decreases lung ERα protein abundance in newborn male rat pups *(p= 0.03),* but not in female rat pups (Figure 7).

ERβ protein relative to GAPDH was measured next. Lung ERβ protein abundance is similar in female and male control newborn rat pups. IUGR increases lung ERβ protein abundance in newborn male rat pups *(p= 0.001),* but not in female rat pups (Figure 8).

Lastly, the protein abundance of AR relative to GAPDH was measured. Lung AR protein abundance is higher in female control than in male control newborn rat pups *(p=0.0393).* IUGR increases in AR protein abundance in male *(p=0.0058)* but not newborn female rat pups (Figure 9).

In summary, the serum and lung estrogen to testosterone ratios as well as ERα protein abundance is depressed in male but not female newborn IUGR rat pups when compared to sex-matched controls. Lung estrogen to testosterone ratio is increased in IUGR females. ERβ and AR expression are increased in IUGR males when compared to sex-matched controls (Table 1).

We conclude that IUGR alters lung sex steroids, estradiol to testosterone ratios, and ER/AR expression in a sex-specific manner in newborn rat lung.

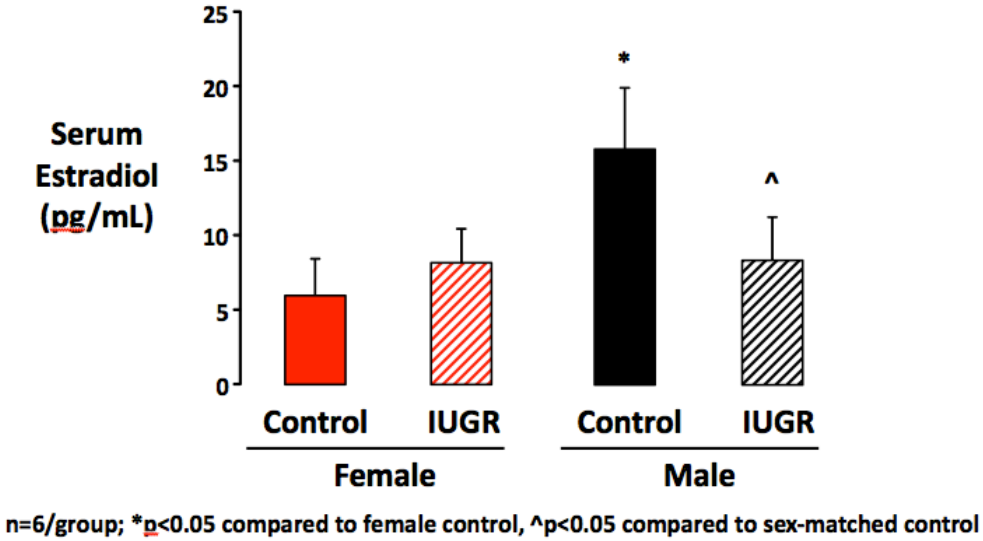

Figure 1. Serum estradiol (pg/mL) in control and IUGR female and male d0 rats

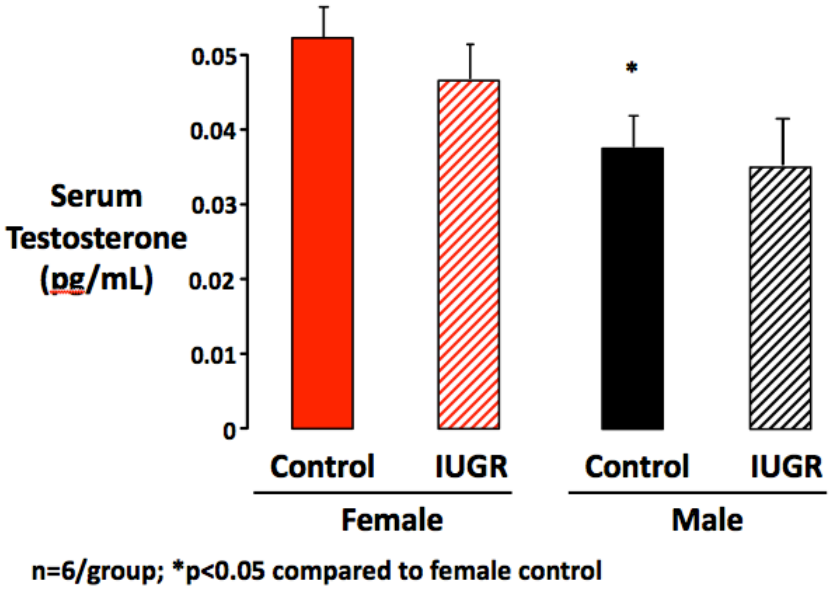

Figure 2. Serum testosterone (pg/mL) in control and IUGR female and male d0 rats

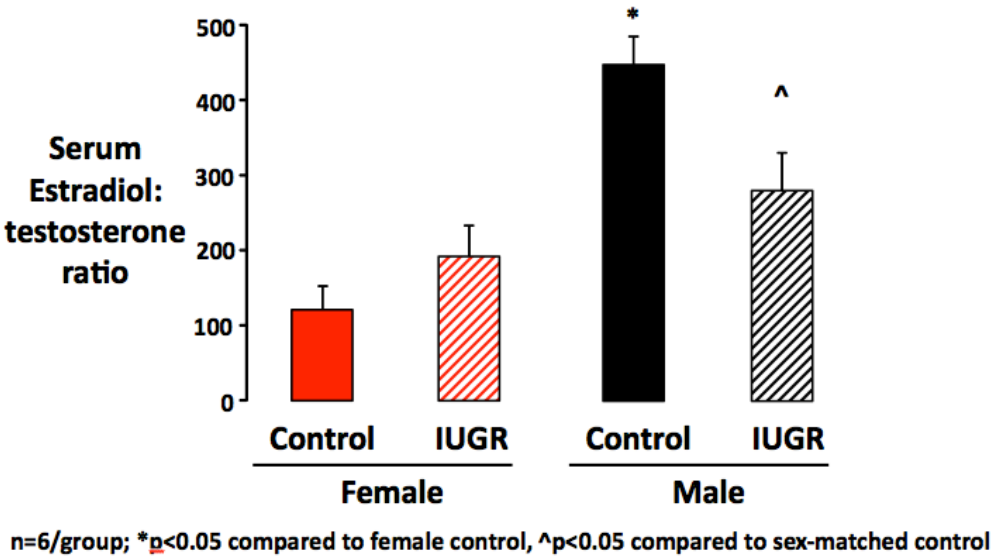

Figure 3. Serum estradiol to testosterone ratio in control and IUGR female and male d0 rats

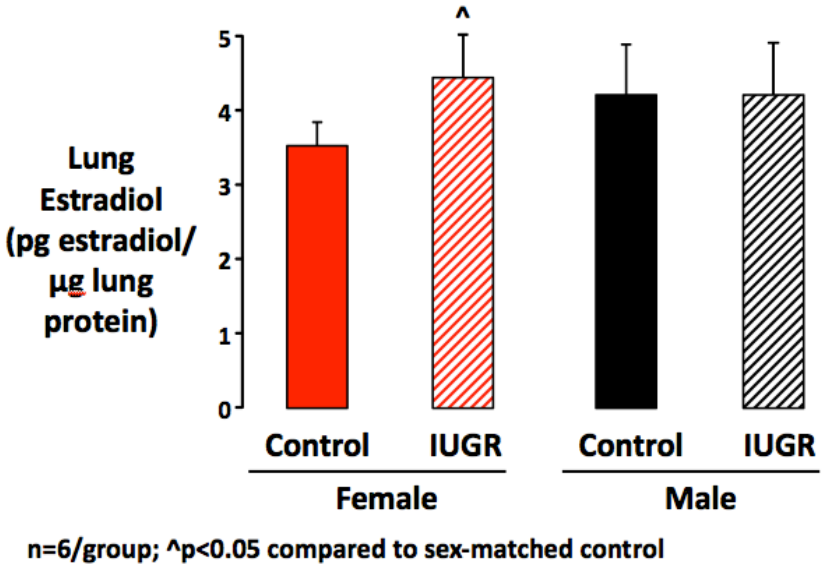

Figure 4. Lung estradiol (pg/µg) in control and IUGR female and male d0 rats

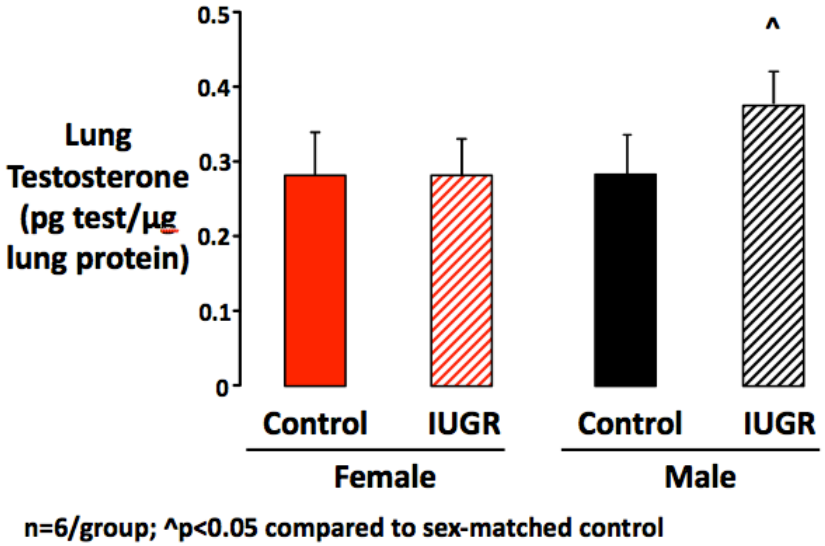

Figure 5. Lung testosterone (pg/µg) in control and IUGR female and male d0 rats

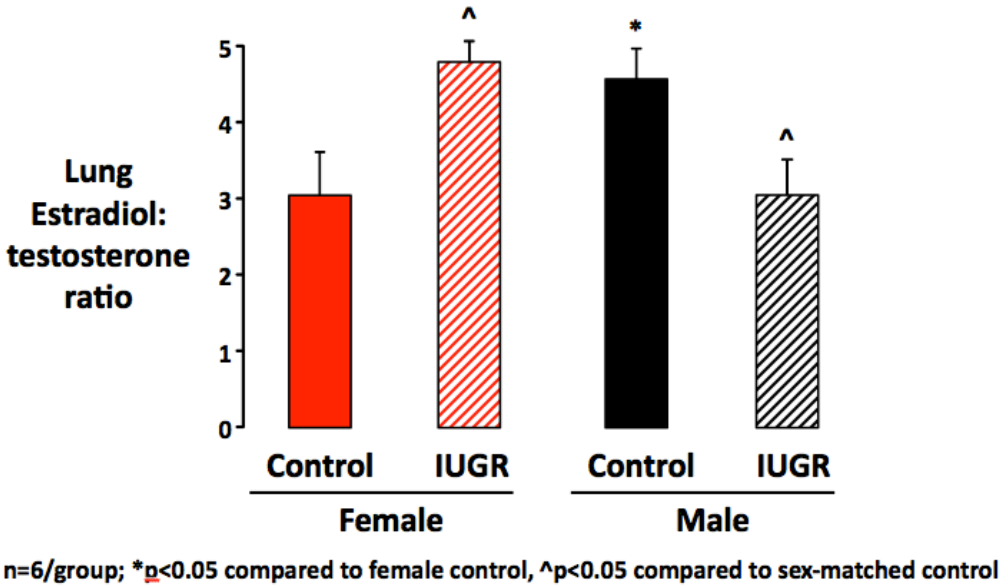

Figure 6. Lung estradiol to testosterone ratio in control and IUGR female and male d0 rats

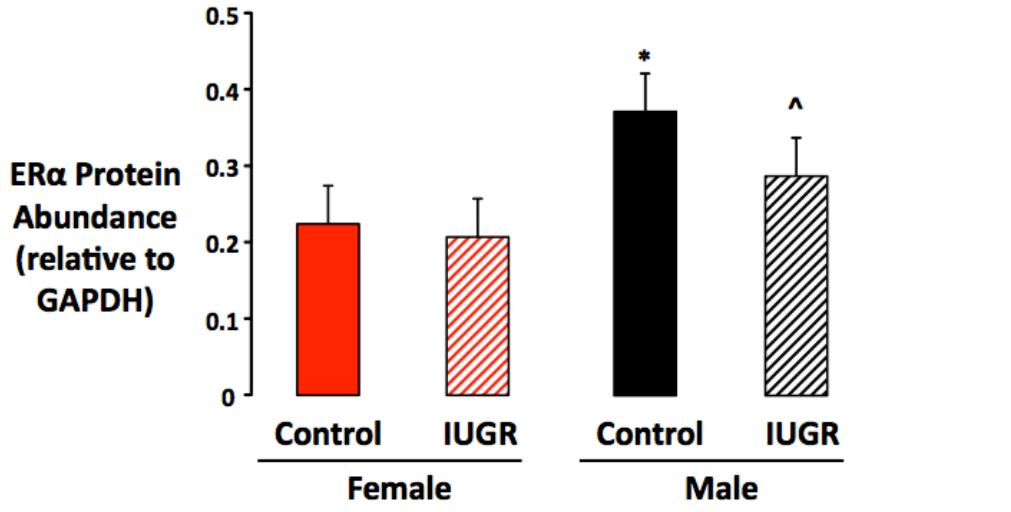

Figure 7. ERα protein abundance in control and IUGR female and male d0 rats

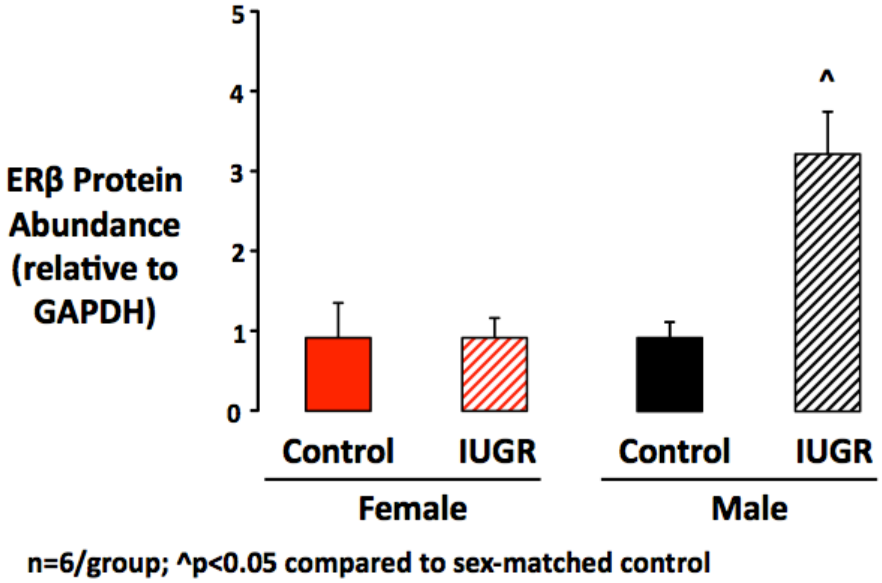

Figure 8. ERβ protein abundance in control and IUGR female and male d0 rats

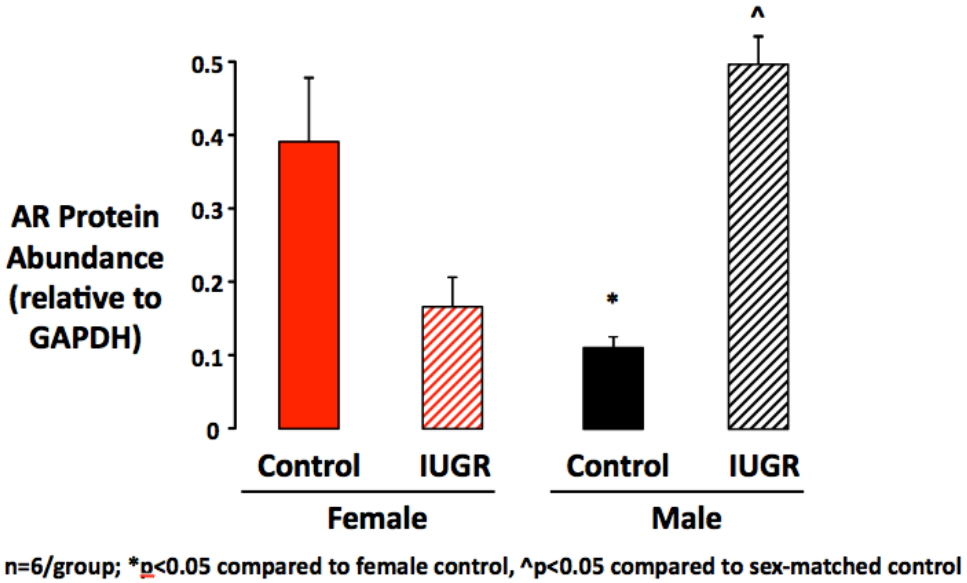

Figure 9. AR protein abundance in control and IUGR female and male d0 rats

Table 1. Summary of sex-divergent results

	IUGR Female	IUGR Male
Lung Est:Test Ratio	↑	↓
ERα Abundance	-----	↓
ERβ Abundance	-----	↑
AR Abundance	-----	↑

DISCUSSION

In this study, we demonstrated that IUGR alters lung sex steroids, estradiol to testosterone ratios, and ER/AR expression in a sex-specific manner in newborn rat lung. Given that IUGR male infants have a higher incidence and a greater severity of BPD than IUGR female infants, understanding the effects of IUGR on sex-steroid signaling in the IUGR lung is important. Our results suggest that IUGR may impair estrogen effects in the lung of male, but not female, rats. These data suggest that altered sex-steroid signaling in IUGR may contribute to worse lung outcomes in males.

In our study, female lung tissue may have adequate ligand and receptor activity to function normally, while male lung tissue may not. The decrease in estradiol to testosterone ratio in IUGR males may contribute to the decreased expression of ERα also observed in the male sex (Stabile, 2002). The AR increase in IUGR males may mirror testosterone concentrations seen in these pups.

The up regulation of ERβ in IUGR male rat pups may seem counterintuitive. ERβ, however, may only function in remodeling of adult lung and may not have an impact on the development of fetal lung tissue (Heldring, 2007). It has also been shown that ERα exhibits higher levels of activation and thus has more impact on downstream targets; its down regulation in IUGR males may have much larger implications on gene regulation than the observed changes in ERβ expression (Hall,

2001). Furthermore, in cells where ERα and ERβ are coexpressed, the ratio of these receptors may be what determines estrogen responsiveness (Hall, 1999).

Some limitations of this study are the inherent difficulties of using an animal model to understand a human pathology. The IUGR rat model we used does, however, produce pups with metabolic similarities to human IUGR infants, and the sequence of events in alveolar formation in the rat is similar to that of humans. The small sample size we used is another limitation, but is necessary to maintain the humaneness of animal studies. Based on our prior studies, an *n* of 6 rat pups per group has provided adequate statistical power to detect a change of ~10% between groups. An additional limitation of our study was that we only measured estradiol (E2). Estriol (E3) is another estrogen, which is highly expressed in rat placenta and may also be biologically relevant.

Strengths of this study include using a well-defined model that accurately mimics human IUGR and examining mechanistically important pathways.

Future research will examine downstream gene targets of ER and AR to complete the signaling pathway data from ligand, through receptor, to target gene. Looking at other estrogens such as E3 could also be beneficial in understanding sex-steroid mechanisms of IUGR developmental divergence.

This research helps us better understand the sex-steroid hormone metabolism and signaling leading from IUGR prenatal insults to later sex-divergent lung development. Eventual conclusions from continuations of this research may lead to the development of targeted interventions.

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